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Enterococcus Spp. in Endodontically Treated Teeth With and Without Periradicular Lesions

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**Enterococcus Spp. in Endodontically Treated Teeth With and Without
Periradicular Lesions.**

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A Thesis

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Requirements for the Degree of

Masters of Science

at the

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APPROVAL PAGE

Master of Science Thesis

**Enterococcus Spp. in endodontically treated teeth with and without
periradicular lesions.**

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Abstract

The purpose of this study was to determine if *Enterococcus* spp. are more prevalent in endodontically-treated teeth with periradicular lesions compared with teeth that require retreatment but have no periradicular rarefaction. Fifty-eight teeth which had received root canal therapy more than one year previously and required retreatment were included.. Designation of lesion vs. no lesion was determined by two experienced endodontists. DNA extraction and PCR amplification were performed using ubiquitous 16S rDNA bacterial primers, as well as *Enterococcus* spp.-specific primers. The results showed that the overall prevalence of bacteria was 90% and *Enterococcus* spp. was 12%. Chi-square analysis revealed a statistically significant relationship between the presence of a lesion and the presence of bacteria, as detected by the universal primers ($p=.032$). Using logistic regression, a statistically significant relationship was found between teeth with normal periapex and the presence of *Enterococcus* spp. ($p=.023$). This study revealed that bacteria are significantly associated with endodontic treatment failure but enterococci are not associated with disease.

INTRODUCTION

Bacterial etiology of periapical lesion

Microbiology and endodontics are intimately related. Microbiological aspects of the root canal space must be considered of equal importance to that of the technical challenges present when instrumenting and filling the root canal space. Without such a blend of the two modalities successful therapy cannot be obtained. However, decisive scientific evidence to support the true importance of bacteria in the development of periapical disease did not exist before 1965 (Takehashi et al. 1965). Prior to this date, only associations had been established between root canal failure and microorganisms. Early pioneers such as Onderdonk recognized bacterial implications and advocated culturing before filling root canals. He also recommended two visits prior to obturation (Onderdonk 1901). In 1906, Buckley, advocated changing a dressing of formocresol several times until no pus was present prior to obturation (Buckley 1906). However, there were still misconceptions regarding the etiology of endodontic failure. Blayney described three causes of failure: introduction of outside infection, traumatization of remaining tissue by careless handling and lastly the use of irritating drugs (Blayney 1927).

Conclusive evidence to support a causative relationship between bacteria and periapical disease was documented in an experiment by Takehashi *et al* (1965). Takehashi demonstrated through an animal model that germ free rats did not develop pulp necrosis or periapical lesions when their pulps were mechanically exposed whereas pulps exposed in rats with a normal bacterial

flora did develop periapical rarefactions. Exposure of the pulp alone did not cause periapical disease. Bacteria were the critical piece needed to trigger the cascade of events that culminates in periapical breakdown (Takehashi et al. 1965).

This study was followed in close succession by Torneck in 1966 who disproved the hollow tube effect proposed by Rickert and Dixon in 1931. He repeated their study with an important modification. He used a rat model instead of a rabbit and instead of placing the hollow tubes in an area of significant friction tubes were placed into the dorsal subcutaneous tissue. The sterile inert polyethylene tubules induced little or no inflammation (Torneck 1966). However, when necrotic tissue inoculated with gram-negative cocci was placed in the tubes severe inflammation with abscess formation was noted (Torneck 1967).

A monkey study demonstrated the influence of indigenous oral bacteria and necrotic pulp tissue on the periapical health (Moller et al. 1981). Seventy-eight teeth were aseptically necrotized. Fifty-two of the 78 teeth were infected with autogenous oral flora while the remainder were left bacteria free. The monkeys were followed for 6 to 7 months. At the conclusion of the study all of the 52 teeth that were infected developed apical periodontitis. The remaining 26 teeth which were still sterile had no signs of periapical inflammation. The conclusion from this study is that sterile necrotic tissue is benign in itself; it is the bacteria that induce periapical breakdown. Dahlen and Bergenholtz also appreciated the importance of certain bacteria in the development of periapical

disease; necrotic tissue alone cannot cause or maintain periapical osteolysis (Dahlen and Bergenholtz 1980).

Origin of endodontic infections

The source of the bacteria became a debate. Through the 1940's and extending into the late 1950's the theory of anachoresis was dominating the field (Robinson and Boling 1941; Gier and Mitchell 1968; Delivanis and Fan 1984). This theory stated that microorganisms reach necrotic pulps via the blood stream. However, several studies disproved this notion (Moller et al. 1981). Many other theories persist regarding how teeth initially become infected; the most obvious one is direct exposure of the pulp to the oral cavity, while other modes of entry include open dentinal tubules, accessory canals or cracks.

With the predominant avenue of bacterial entry into the pulp via caries and exposed dentin established the next most important question is which bacteria comprise the primary infection. Initially an exposure of the surface region of the pulp to the oral cavity will result in a surface infection predominated by aerobic bacteria. The first bacteria to invade the pulp are gram positive. However, gram negative facultative anaerobes increase in number over time and outnumber the aerobic population (Sundqvist 1992). A continuum exists whereby the flora begins to shift in the apical region in a closed environment. A decrease in oxygen tension selects for obligate anaerobes (Sundqvist 1992) and as the periapical disease progresses the flora of the untreated tooth is comprised of approximately

90% strict anaerobes (Sundqvist 1976; Bystrom and Sundqvist 1981; Haapasalo 1989; Sundqvist et al. 1989).

Bacterial species associated with periapical pathology

Although many bacteria are able to stimulate periapical inflammation certain bacteria and combinations of bacteria are more potent. In an attempt to prove Koch's postulate, Sundqvist and co-workers found that only a combination of bacteria isolated from an infected root canal not a single species could induce transmissible infection in an animal model (Sundqvist et al. 1979). Transmission was further facilitated by certain species present in the samples. Successful transmission was achieved when combinations of *P. intermedia* or *P. endodontalis* were present (Sundqvist et al. 1979). Thus, through successful transmission one of Koch's postulates was fulfilled. Sundqvist's findings have been replicated by others (van Winkelhoff et al. 1985). In addition to *P. intermedia* and *P. endodontalis*, *P. gingivalis* was also found to be a major component in the mixed infection seen in endodontic abscesses (van Winkelhoff et al. 1985).

There is also a certain profile of bacteria associated with exacerbations and drainage. *Porphyromonas* species are associated with development of exacerbations. In an in vivo study of 72 teeth with periapical rarefactions 17 cases had exacerbations. Among those 17 cases with acute abscesses 90% were associated with recovery of *Porphyromonas* (Sundqvist et al. 1989).

A relationship exists between the number of strains found in a root canal and symptomatology. In a clinical study of 16 necrotic teeth with intact crowns all

7 painful cases had six or more species present within the root canal (Sundqvist 1976). The 9 cases without pain had fewer than 6 species present within the root canal space. There also seems to be an association between lesion size and the number of strains present. Of the 9 teeth with a periapical lesion greater than or equal to 5mm, 8 of them had at least 5 strains present. Likewise, 8 of the 9 teeth with lesions measuring less than 5 mm had fewer than 6 strains of bacteria present (Sundqvist 1976). Thus, it would seem, a threshold that must be reached where a certain bacterial load or quantity of bacterial strains must be present to induce significant osteolysis and clinical symptoms.

Therapeutic endpoint

Eradication of the bacteria within the root canal space has posed a formidable challenge. Calcium hydroxide has been shown to eliminate most microorganisms when used as a 7-day dressing (Sundqvist et al. 1998). A comparison between the antimicrobial effects of calcium hydroxide and iodine-potassium iodide (IKI) in a clinical study revealed that fewer culture reversals occurred when calcium hydroxide was used compared with IKI (Safavi et al. 1985).

If these bacteria are not eradicated during the initial instrumentation and root filling process, the long-term prognosis decreases (Engström et al. 1964; Heling and Shapira 1978; Sjögren et al. 1997). Engström followed 306 cases treated by students in which root canals were performed. Prior to obturation cultures were taken. The observation period was between 4-5 years. He found

that teeth without pre-operative radiolucencies and with a negative culture at the time of obturation had the best prognosis. The success rate for this group reached 96.1%. The prognosis dropped to 87% if the tooth without a pre-operative radiolucency had a positive culture. The group with the worst prognosis were those teeth with both a pre-operative lesion and bacteria present. The success rate among those teeth was only 62.1%(Engström et al. 1964).

Heling had a similar study, where he treated 118 teeth with repeated cultures taken prior to obturation. Teeth were followed from 1-5 years. The mean success rate for root canal treatment was 78%. Teeth that had a negative culture prior to obturation had an 80% success rate while teeth with a positive culture had a 70% success rate. Interestingly, sterility of the root canal influenced the rate of success as time increased. The rate of success was 74% for teeth with a negative culture at recalls performed 1-3 years post obturation. The success rate dramatically increased at the 4-5 year recall to 86% for teeth with a negative culture. This is in contrast to the group of patients with a positive culture at the time of obturation, the success rate of those teeth decreased from 73% at the 1-3 year recall to 60% at the 4-5 year recall.

The early studies by Engström *et al.* and Heling *et al.* utilized a traditional culture technique as a means of identifying if bacteria were present while Sjögren used an advanced anaerobic sampling technique of identifying bacteria. Despite the more advanced identification technique the results borne out in the Sjögren data echo those of Engström and Heling. Sjögren found that 5 years post obturation teeth that were free of bacteria at the time of root filling had a success

rate 26% percentage points higher than those that had bacteria present at the time of root filling (Sjögren et al. 1997).

There is considerable literature to substantiate the effect of root filling length on prognosis. Sjögren found that slight overfills had no influence on outcome. All of the overfills in his sample were successful at the 5 year follow-up (Sjögren et al. 1997). However, Heling showed that overfills had the lowest success rate irregardless of bacteria presence at the time of obturation. Success rate of teeth overfilled was 73%. Obturations flush and underfilled had a success rate of 80 and 82%, respectfully (Heling and Shapira 1978). A subtle distinction may exist regarding the importance of overfills on prognosis, this is most clearly evident in Stringberg's thesis where he distinguishes between slight and substantial overfills. He found that teeth with a pronounced overfill had a significantly lower proportion of successful root fillings than teeth with only a slight overfill at 4 years follow-up (Strindberg 1956).

The work by Engström highlights that overfills are particularly detrimental if bacteria are present at the time of obturation. He found the success rate for teeth with an overfill and bacteria present was only 58.6% If the obturation was flush or short but bacteria were present the prognosis rose to 78.8%. If the overfill was done aseptically and no bacteria were present at the time of obturation than a success rate of 90% was possible (Engström et al. 1964).

Sjögren looked at the importance of length on both necrotic cases and retreatment cases (Sjögren et al. 1990). He found necrotic teeth obturated between 0-2 mm of the radiographic apex had the highest success rate. Success

rate dropped from 94% to 76 % if extrusion was present and dropped even further to 68% if the obturation material was more than 2 mm short. A similar pattern existed among retreatment cases. Obturation cases 0-2 mm of the radiographic apex had a success rate of 67% while teeth with extrusion had a 50% success rate and those >2 mm short had 65% success rate (Sjögren et al. 1990). Thus it can be surmised that obturations 0-2 mm short of the apex are optimal. Deviations from the ideal decrease prognosis especially if bacteria are present.

Persistent infection following root canal preparation has been considered the main etiologic factor in root canal failure (Sjögren et al. 1997; Sundqvist et al. 1998). Some have also speculated that re-infection via coronal microleakage may also contribute to failure of endodontic treatment (Ray and Trope 1995; Fouad and Burleson 2003). Investigators have examined the bacterial profile of root-filled teeth with periradicular periodontitis in an attempt to isolate the primary microorganisms responsible for these failures. When the bacterial flora of root filled teeth with periradicular rarefactions is compared to untreated teeth with apical pathosis, the profiles are remarkably different (Sundqvist et al. 1998). Teeth with primary infection have higher numbers of black pigmented Gram-negative obligate anaerobes (Sundqvist 1994), while root filled teeth with periradicular lesions have significantly fewer black pigmented rods and more Gram-positive facultative anaerobic bacteria. Specific culture assays have found that the bacterial species *Enterococcus faecalis* is present in root filled teeth with periradicular periodontitis approximately 37% of the time (Sundqvist et al. 1998).

Table 1 shows a complete list of culture studies that report on the frequency of *E. faecalis* in retreatment cases with apical pathology.

More sensitive polymerase chain reaction techniques have just recently reported the frequency of *Enterococcus faecalis* in root filled teeth with periradicular rarefactions to be as high as 77% (Siqueira Junior 2004). However, this high recovery rate may be an outlier among the majority of studies. Rolph et al. showed that *Enterococcus* spp. were not recovered within their sample of 11 retreatment cases (Rolph et al. 2001). Likewise, recent data from our lab revealed a prevalence of 22% among retreatment cases (Fouad and Barry 2005). See Table 2 for a complete list of molecular studies and their reported frequencies of *Enterococcus* recovery among therapy resistant cases.

If *Enterococcus faecalis* is associated with a higher incidence of root canal failure then eradicating these bacteria and preventing them from repopulating obturated root canals would be a primary objective of retreatment. Studies have shown that *Enterococcus faecalis* is relatively resistant to many conventional intracanal medicaments. Calcium hydroxide, a standard intracanal medicament, has proven to be ineffective at completely eradicating this bacterial species (Orstavik and Haapasalo 1990; Chavez de Paz Villanueva 2003). Calcium hydroxide exerts most of its antimicrobial effect through its alkalinity (Tronstad et al. 1980). The high pH destroys cell membranes and is not compatible with bacterial viability over a pH of 9.5 (Fisher and Huerta 1984). However, *E. faecalis* has the ability to withstand a pH of 11.1 (Evans et al. 2002). It was initially thought that there was an inducible stress response mechanism that provided

protection for *E. faecalis* to calcium hydroxide. It was believed that an initial exposure of calcium hydroxide was necessary to confer protection at subsequent exposures. However, it was shown that pre-treatment with calcium hydroxide pH 10.3 did not induce tolerance to further exposure at pH 11.5 (Evans et al. 2002). Blockage of protein synthesis did not affect cell survival. However, addition of a proton pump inhibitor produced dramatic reduction in *E. faecalis* cell survival in the presence of calcium hydroxide. The believed mechanism for evasion of the killing effect of calcium hydroxide is a proton pump that acidify the cytoplasm and allows *E. faecalis* to live in high pH environments (Evans et al. 2002).

Attempts have been made to use less conventional medicaments and irrigants such as Chlorhexidine (CHX) (Zerella 2003; Siren 2004), Iodine potassium iodide (IKI), tetracycline (Dahlen et al. 2000), ozone (Hems et al. 2005) and the recently-described mixture of tetracycline analogue, citric acid, and detergent (MTAD)(Shabahang 2003). While calcium hydroxide alone appears to be ineffective at eradicating *E. faecalis* in vivo, researchers have hypothesized that adding additional agents such as antibiotics, shown to have effectiveness against these bacteria, may be a logical solution. Calcium hydroxide when used as a dressing has been shown to eliminate most microorganisms (Sundqvist et al. 1998) and therefore would be a valuable medicament to continue to use in the root canal system. The addition of erythromycin targeted specifically for *E. faecalis* may represent a comprehensive approach. An in vivo study measuring the effectiveness of tetracycline or erythromycin mixed with calcium hydroxide found antimicrobial effectiveness against *Enterococcus* to be 79% and 96%,

respectively. However, the total antimicrobial effect against the mixed infection present within the root canal was considerably lower. The tetracycline mixture had only a 54% effectiveness and the erythromycin mixture only 56%. Thus, the overall antimicrobial effect of these agents was weak although erythromycin may show promise as a medicament to target monoinfections of *Enterococcus* (Molander and Dahlen 2003).

A recent study explored an antimicrobial agent not traditionally used in contemporary endodontics, ozone, which is a powerful oxidizing agent. It has been used as a hospital disinfectant and at water treatment facilities to kill bacteria. An in vitro model to simulate a planktonic and biofilm infection comprised of *Enterococcus faecalis* was utilized. Although the ozone did reduce viability of *E. faecalis* in the planktonic suspension it had little effect on bacteria in the biofilm (Hems et al. 2005).

A group in New Zealand has also explored additional antibacterial agents not currently being used in endodontics. They have turned their attention to nisin which is a naturally occurring antimicrobial peptide and has been used extensively in food preservation. It is an interesting candidate because it has a benign effect on humans but also has a detrimental interaction with the phospholipid membrane of target bacterial cells. In an in vitro model designed to simulate radicular infection the antimicrobial effect of nisin was evaluated compared to conventional calcium hydroxide. Nisin was effective at eradicating *E. faecalis* in pure cultures, however, it only reduced *E. faecalis* in canal wall

radicular dentin by 48%; calcium hydroxide had a similar result only reducing *E. faecalis* by 45% (Turner et al. 2004).

Some practitioners have incorporated iodine into their arsenal of antimicrobial irrigants. Iodine has the advantage of having low tissue toxicity. It exerts its antimicrobial effect by acting as an oxidizing agent and reacting with sulfhydryl groups of bacterial enzymes resulting in disulfide linkages. The major disadvantage of iodine is allergic reactions in some patients. Iodine does not lend itself to placement as an intracanal medicament due to its short duration of antimicrobial action. Iodine has shown considerable promise as an antibacterial agent targeted toward *E. faecalis* eradication. Peciuliene and coworkers demonstrated that when a chemo-mechanical preparation is followed by a 5 minute flush of IKI 87.5% of teeth, which initially tested positive for enterococcus, were rendered free of cultivable enterococcus (Peciuliene et al. 2001).

Some have postulated that the addition of a surfactant to iodine products may improve on the antimicrobial actions by reducing the surface tension. When 2% iodine potassium iodide (IKI) was compared with Betadine scrub, a povidone-iodine solution with surfactant, using a modified Haapasalo and Orstavik dentinal infection model, both IKI and Betadine Scrub used as a 24 hour dressing were able to render 90% of enterococcus infected samples sterile. However, Betadine was significantly less effective as a 15 minute irrigant than IKI (Baker et al. 2004). Thus, addition of surfactant did not improve the efficacy of iodine containing agents against in vitro enterococcal infections.

The multitude of agents researchers have tried to eliminate this bacteria underscores the tenacity of *E. faecalis* to persist in the root canal system.

Role of *E. faecalis* in the pathogenesis of periapical lesions

The available data remain equivocal as to whether *Enterococcus* spp. are causative of endodontic failures, or opportunistic bacteria that populate the treated root canal because the environment is conducive to their survival. In this regard, there is little clinical data available regarding the prevalence of *Enterococcus* spp. in root filled teeth without periradicular rarefactions. Molander and coworkers (Molander et al. 1998) have been the only investigator to look at the microbiologic status of root filled teeth with normal periapices. He examined the microbiological status of 120 root-filled teeth: 100 with periradicular periodontitis and 20 with normal periapices. In this study, the prevalence of *Enterococcus* spp. in root filled teeth with apical lesions and those root filled teeth with a normal periapex was thirty-two and five percent respectively. While this study stands alone as a predictor of the true bacterial profile of root filled teeth without periradicular rarefactions, the study design has several weaknesses. The use of chloroform prior to bacterial sampling has been shown to reduce bacterial detection (Molander et al. 1998; Fouad and Barry 2005). Furthermore, 26.6% of the teeth with lesions in the Molander *et al.* study had no bacterial growth, indicating the low sensitivity of the culturing techniques used. In addition, a high percentage of lactobacillus were recovered among the positive cultures which may represent contamination.

Thus there appears to be scarce evidence that *Enterococcus* species are truly unique to the failing root filling and not merely a commensal part of the root filled tooth. Therefore, it may be premature to focus antimicrobial therapies on eradicating one bacterial species which may not be solely responsible for endodontic pathosis. The hypothesis for this study is that *Enterococcus* spp. have a higher prevalence in root canal treated teeth with periradicular lesions than those without periradicular rarefaction. If this hypothesis is shown to be true, then this microorganism would be a primary suspect in the pathogenesis of periradicular lesions, thereby causing endodontic failure.

MATERIALS AND METHODS

Patient Selection:

All patient procedures were approved by the IRB #04070 at University of Connecticut Health Center, and all patients provided written informed consent prior to being recruited in the study. Two groups of patients were recruited. The first group of patients had an endodontically-treated tooth with radiographic evidence of periradicular periodontitis requiring endodontic retreatment; the second group had an endodontically-treated tooth requiring retreatment but without radiographic evidence of apical pathosis. Cases in the second group were retreated due to suspected coronal leakage or prior to an extensive restoration when the technical quality of the previous treatment was questionable. Suspected coronal leakage and/or poor technical quality of

previous treatment were also frequently present in the first group, except that the teeth in that group also had lesions. Teeth were included in the study only if the initial root filling was completed at least one year previously. Only permanent teeth of adult patients were included.

The following variables were registered for each patient: 1) Peri-operative signs and symptoms: defined as moderate to severe pain to percussion or palpation, or any flare-ups where the patient returns for unscheduled appointments with symptoms; 2) Quality of previous root filling; 3) Quality of the coronal restoration and 4) History of diabetes mellitus. The quality of the previous root filling was dichotomized into: acceptable (obturation 2 mm short of the apex or 0.5 mm long, with no voids more than 1mm) or unacceptable. Likewise, the quality of restoration was dichotomized such that acceptable were only those permanent restorations with adequate seal determined clinically and radiographically.

Sampling Procedure

Teeth were isolated using rubber dam and accessed. Disinfection with 30% hydrogen peroxide and 5% iodine tincture were carried out according to the protocol outline by Möller (Moller 1966). The teeth were further disinfected with 6% sodium hypochlorite (Ng 2003). If the tooth had caries, the disinfection steps were repeated and new sterile instruments were used following caries excavation. Sodium hypochlorite was neutralized with 5% sodium thiosulphate. At this point, a surface specimen was collected with paper points that was to be

analyzed in case of a positive *Enterococcus* spp. result (see below). Root-filling material was removed using Gates Glidden drills, Hedstrom files and Profile® rotary instruments with no solvent. Working length was estimated using a Root ZX and confirmed radiographically. All attempts were made to instrument canals to a length 0.5-1 mm short of the radiographic apex. Saline was introduced into the canal, once an apical size of 25 had been reached and most of the root filling material had been removed. The specimens were obtained using three paper points placed in the canal for about 30 seconds in succession. In addition, a new sterile #25 H-file was used to length, and the working area of the file aseptically separated and added to the specimen. For multi-canal cases, the sampling was performed from one canal and in the case of a tooth with a periradicular lesion the sample was taken from the root with the periradicular rarefaction. The paper points and file were transferred to sterile, DNA/RNA-free vials containing 1mL filter-sterilized 10mM Tris-HCl; 1mM EDTA (pH =8) and 0.5g (0.71-1.18mm) sterile glass beads. The vials were frozen at – 70° C until used.

DNA extraction:

The specimens were thawed and the vials with paper point specimens were vortexed for 2 min to disperse microbial cellular material into suspension. The suspension was transferred from the original vial to 2 ml DNA/RNA-free sterile vials, which were centrifuged for 10 minutes at 7500 rpm, and the supernatant was removed. From the cellular pellet, DNA was extracted by the enzymatic extraction method, using the Qiagen-QIAamp DNA mini kit protocol (Qiagen, Valencia, CA). This was performed after suspending the pellet in 180 µL

of enzyme solution (20 mg/ml lysozyme; 20 mM Tris HCl, pH 8.0; 2 mM EDTA; 1.2% Triton), incubating it for 30 min. at 37°C, and adding Proteinase K (20 µL) and Rnase A (4 µL at 100mg/mL) for 2 minutes at room temperature.

PCR primers:

All specimens were amplified with ubiquitous (broad range) bacterial primers that target the 16S rRNA gene to determine bacterial presence, as described previously (Fouad et al. 2002). The following primers were used for this purpose: Forward: AGA GTT TGA TCC TGG CTC AG, and Reverse: ACG GCT ACC TTG TTA CGA CTT. In addition, genus-specific oligonucleotide primers were used to identify the *Enterococcus* spp. The following primers, which amplify a 112 bp sequence (Ke et al. 1999), were used to amplify enterococcal DNA sequences in the *tuf* gene: Forward: TAC TGA CAA ACC ATT CAT GAT G and Reverse: AAC TTC GTC ACC AAC GCG AAC. These primers have been shown to detect 14 species within the genus *Enterococcus* (Ke et al. 1999), and two *Abiotrophia* spp. were shown to be negative with 17 other endodontic pathogens (Fouad et al. 2002).

PCR Assay:

PCR amplification was performed in a PE9700 thermal cycler (Applied Biosystems, Foster City, CA). PCR conditions were generally as follows: the initial denaturation was at 94° C for 15 min. This was followed by 35 cycles of denaturation at 94° C for 15 s, annealing at 55° C (56° C for ubiquitous primers) for 15 s and extension at 72° C for 45 s. The final extension was at 72 C for 5

min, then the products were cooled to 4° C until removed (Fouad et al. 2002). The reaction was carried out in a volume of 50 µL containing 5 µL of extracted sample DNA, 5 µL of 10x PCR buffer, 0.5 µL HotStar Taq, (Qiagen, Valencia, CA), 1.5 mM MgCl₂, 0.2 mM of each of the 4 deoxynucleoside triphosphates (dNTP) (Takara, Otsu, Shiga, Japan), 0.5 µM (500 ng) of each (sense and antisense) primer and the balance made up of sterile ultrapure water. All PCR experiments had positive (extracted *E. faecalis* DNA) and negative (ultrapure water) controls. Amplification products were analyzed by 2% agarose gel electrophoresis in Tris-acetate EDTA (TAE) buffer (40 mM Tris-acetate, 2 mM EDTA, pH 8.3). Gels were stained with 0.5 µg/mL ethidium bromide for 30 minutes and destained with water for 15 minutes. The PCR products were visualized under UV light.

Direct Sequencing of PCR products:

In order to verify that the positive specimens did contain *Enterococcus* spp., and to determine which species were present, direct sequencing was performed on the *Enterococcus*-positive PCR products (Fouad 2003). For sequencing, the PCR products were reamplified, then purified using Marligen Bioscience Rapid PCR Purification System (Marligen Bioscience, Inc., Ijamsville, MD.) Sequencing reaction mixes were prepared with forward and reverse *Enterococcus* primers, separately (11-32 ng DNA per reaction mix). Sequencing was performed by the Molecular Core Facility, at the University of Connecticut Health Center, using the ABI Prism 3100 Genetic Analyzer. Sequences were used to search databases available through NCBI, using the BLAST algorithm,

then aligned and phylogenetically analyzed using MacVector 7.2 (Accelrys, San Diego, CA).

Data Analysis:

Designation of Lesion vs. no lesion:

Two experienced endodontists who were not involved in the treatment of the patients and who did not know the microbiological results reviewed periapical radiographs individually and rendered a decision of lesion or no lesion. In cases where there was a discrepancy between reviewers both individuals conferred together and a consensus was reached.

Prevalence:

The prevalence of bacteria and of *Enterococcus* spp. were recorded as the percentage of the cases examined. The prevalence values in cases with and without periradicular lesion were compared using the Chi square analysis. A significance level of $p > .05$ was used.

Sample size Determination:

A statistical power analysis had been performed to determine the optimal sample size. The prevalence of enterococci in cases with lesions was assumed to be about 70% based on recent data(Siqueira Junior 2004). Due to the scarcity of data on enterococci in cases with no lesions, we used a figure that was half the number of expected prevalence for cases with lesions, with an assumption that this difference would be clinically significant. Assuming a standard Type II

error rate of $\beta = .20$ (power = $1 - \beta = .80$), and a standard Type I error rate of $\alpha = .05$, the effect size was calculated to be 0.72. According to Cohen's textbook (Cohen 1988) a sample size of $n = 24$ per group would be needed to yield statistical power of 80%. We recognized that it was more difficult to recruit patients in the no lesion category than cases with lesions, and made an assumption based on the previous year's treated cases that there will be a 2:1 ratio between the sample sizes of the Lesion versus the No Lesion groups. Therefore we used $n' = 24$ as the harmonic mean of the groups needed to meet the criteria. The formula for $n' = 2(n_1)(n_2) / (n_1 + n_2)$, was used to determine the sample needed in the No Lesion group (n_1), and Lesion group (n_2). This yielded a sample size of $n=18$ for the No Lesion group and $n=36$ for the Lesion group, for a minimum total $n = 54$.

RESULTS

Sixty-five patients were recruited for the study; however, seven of those patients had been on antibiotic during some portion of the preceding two months so they were excluded from the analysis. Thus the final sample size was 58, in which 22 cases had no periradicular lesions and 36 were with lesions.

Overall, bacteria were detected in 52 of 58 cases (89.7%). They were present in all but one case in which lesions were present, and in 17 of 22 cases with no lesions (Table3) (odds ratio=10.3). Chi-Square analysis revealed a statistically significant relationship between lesion status and the presence of bacteria using the universal primers ($p=0.032$). Teeth with a periradicular lesion

were significantly more likely to have bacteria than teeth without a periradicular lesion.

Additional parameters were also evaluated. Restoration quality was not associated with lesion status (Table 4). Likewise, obturation quality was not associated with periapical health (Table 5).

Enterococcus spp. was present in only 7 of 58 cases or 12.1%. Five cases with *Enterococcus* spp. were in cases with no lesions, while only two were in cases with lesions (Table 6)(odds ratio = 5). A 2-sided fisher's exact test revealed a trend toward significance ($p = 0.10$).

Seven of the 58 cases included in this study were from diabetic patients; one from type 1 and 6 from type 2 diabetics. Four of these were in the non-lesion and three in the lesion groups. All cases from diabetic patients had bacteria detected in the root canal, but none of them had enterococci. Table 6 shows the other clinical parameters associated with the cases with or without enterococci. A logistic regression analysis was performed to control for the quality of restoration, quality of obturation, peri-operative symptoms, and history of diabetes. There was a statistically significant inverse relationship between presence of *Enterococcus* and lesion status ($p = 0.023$). *Enterococcus* spp. was associated with non-lesion teeth. Controlling factors failed to predict likelihood of *Enterococcus* spp. alone or collectively but when controlling for them, they allowed lesion status (lack of a demonstrable lesion) to be a strong predictor of *Enterococcus* spp. recovery. Using sequential entry of independent variables none of the four independent variables (the quality of restoration, quality of

obturation, peri-operative symptoms, and history of diabetes) was shown to be statistically significantly related to the dependent variable, recovery of *Enterococcus* spp., nor were they collectively able to account for the presence of a periradicular lesion.

The type of obturation material encountered upon retreatment was also recorded. All of the teeth treated had been obturated with gutta percha except one that contained a silver point. *Enterococcus* was not recovered from the patient sample containing a silver point. Only four patients presented with preoperative sinus tracts, therefore, these two variables were not used in the statistical analysis.

The phylogenetic analysis of the identity of the seven positive specimens revealed that they formed a clade that had its closest match with *E. faecalis* V583 (AE016947) (Fig. 1). Examination of the control external specimens from the seven *Enterococcus*-positive canal specimens revealed a positive reaction in only one of them.

DISCUSSION

This study showed that the prevalence of *Enterococcus* spp. in the root canals of cases receiving endodontic retreatment was 12.1%. This prevalence is lower than that previously reported in some studies 64-77% (Rocas and Jung 2004; Siqueira Junior 2004) using PCR identification, but is consistent with the 22% reported in an earlier study by our group (Fouad and Barry 2005). In fact an earlier molecular study (Rolph et al. 2001), and one culture study (Cheung and Ho 2001) failed to identify enterococci in retreatment cases. Enterococci are

capable of surviving for long durations under adverse circumstances (Figdor 2003), and in the treated root canal environment their presence may be related to their overall presence in the mouth(Engstrom 1964). Certainly, many cases with lesions had bacteria in the root canals, but no enterococci.

The identity match of the enterococci sequenced in this study were similar to those identified in our previous study, in which the same molecular detection techniques were used(Fouad and Barry 2005). The elongation factor Tu (*tuf*) gene is not as well characterized as the 16S gene to be used for bacterial identification in general, but can be used to screen sequences within a smaller group of microorganisms. The prevalence of *Enterococcus* spp. in this study, however, was lower than the 22% reported in our previous study of 37 retreatment cases with periradicular lesions(Fouad and Barry 2005). The difference between the overall prevalence of this microorganism in the two studies was not statistically significant (Chi-square; $p=0.213$). However, if only the cases with lesions in this study are considered ($n=36$, with two specimens positive for *Enterococcus* spp.), then the difference between the studies is significant (Chi-square; $p=0.046$). This apparent difference between the studies may be related to the fact that in the previous study the microbial specimens were incubated in thioglycollate broth prior to performing the molecular techniques. This may have allowed more enterococci to grow and thus improved the overall detection sensitivity. However, this difference is not relevant to the main findings of this study, since the same techniques were used for both groups examined.

Molecular studies cannot be directly compared, particularly if different oligonucleotide primers are used, due to potential differences in primer sensitivity or optimization of reactions. Thus, the differences between the findings of this study and the high values reported previously (Rocas and Jung 2004; Siqueira Junior 2004) could be due to differences in primer sensitivity. Therefore, a pilot study was performed prior to analysis of patient samples. Known concentrations of bacterial cells of an ATCC stock strain of *E. faecalis* (19433) were analyzed using the PCR primer published in those studies and the primers used in this study. The primers, used in this study, were found to be 10-fold more sensitive than those reported previously. Thus, primer sensitivity could not explain the differences in recovery rates between this study and those reported previously.

It is possible that the differences seen in prevalence of this microorganism are due to geographical differences in the prevalence of enterococci in the mouth, a factor that was recently recognized in the endodontic literature (Baumgartner and Siqueira Junior 2004). In any case, the design of this study was such that primer sensitivity was an irrelevant factor since all specimens from cases with and without lesions were analyzed using the same conditions.

Unlike culture modalities, PCR measures the presence of DNA. Therefore, disinfection procedures for PCR sampling are unique compared to those for culture. Complete removal of all traces of DNA is a formidable challenge. Very little has been written about definitive disinfection procedures for reliable removal of DNA. An in vivo study of decontamination of the operating

field using either 10% iodine or 2.5% NaOCl revealed a recovery rate of bacterial DNA using PCR in 45% and 13% respectively (Ke et al. 1999). Thus an effort was made in our experiment to improve disinfection procedures and minimize contamination by using a higher concentration of NaOCl 6% in addition to iodine and H₂O₂. Despite, our strict aseptic technique and our thorough disinfection process one of the control samples taken from the chamber revealed enterococcal DNA. This tooth also tested positive for *Enterococcus* within the canal system. Hence, the uncertainty exists how much contamination exists in studies where weaker dilutions of NaOCl and no iodine were used. Clearly more studies will be needed to elucidate the answer.

One of the observations of this experiment was a statistically higher recovery of bacteria, using a broad range 16S bacterial primers, in teeth with periradicular pathosis. This is consistent with the established belief that periapical disease is due to a bacterial etiology (Kakehashi et al. 1965; Sjögren et al. 1997).

It has recently been speculated that *Enterococcus* spp. may play a role in endodontic failure due to its low recovery in primary infections and higher recovery in secondary infections (Molander et al. 1998). However, the results of this experiment suggest otherwise. Surprisingly, when possible confounding factors were controlled for, there was a statistically higher number of teeth without periradicular lesions which were found to harbor this bacteria compared to teeth with periradicular lesions. One explanation might be that the teeth without periradicular pathosis might be in the process of developing a periradicular lesion. These explanations were accounted for in the study design.

Non-lesion teeth were included in the study only if obturation had been completed more than one year previously. The range of time from obturation to retreatment for patients who tested positive for *Enterococcus* spp. was between 2 and 28 years (mean=11.7 years.)

The results of this study are strengthened by the large sample size obtained in comparison to the only other published molecular studies measuring the incidence of *Enterococcus* in retreatment cases. Our sample size was 58 compared to 37 in the Fouad study (Fouad and Barry 2005), 22 in the Siqueira study (Siqueira Junior 2004), 14 in the Rocas study (Rocas and Jung 2004) and only 11 in the Rolph study (Rolph et al. 2001). A power analysis was performed prior to the initiation of our study to insure an adequate sample size would be recruited to effectively draw clinically relevant conclusions.

Enterococcus faecalis is a normal intestinal organism and may inhabit the oral cavity and gingival sulcus (Engstrom 1964; Sedgley and Lennan 2004). In its intestinal environment, it is considered a commensal organism that contributes to carbohydrate, amino acid and vitamin metabolism. However, a subset of this species appear to be pathogenic because they have acquired a number of genes conferring infectivity and virulence, including resistance to multiple antibiotics (Gilmore 2003). The high-molecular weight surface protein Esp, has been found in high frequencies among enterococcal isolates associated with urinary tract infections and endocarditis (Shankar 2001). This surface antigen may aid in the ability of this bacteria to adhere to the lining of the heart and form biofilms. Production of other factors such as cytolysin, hyaluranidase and gelatinase have

been linked to host tissue damage(Kayaoglu 2004). It is known that Enterococcus can cause serious disease processes in certain areas of the body while in others exist without detriment. The presence of this bacteria within the oral cavity is not associated with disease in itself and it is becoming more evident that its role in periapical disease may be minor.

A direct correlation has been demonstrated between the occurrence of enterococci in the oral cavity and pulp cavity (Engstrom 1964). The same bacterial type cultivated from proximal spaces consistently matched those recovered from the tonsil and root canal samples in the same patient (Engstrom 1964). Although *E. faecalis* is found within the oral cavity its recovery rate differs between populations of individuals. Oral rinse samples, obtained from 100 patients receiving endodontic therapy and 100 dental students with no history of endodontic treatment, revealed *E. faecalis* to be present in 11% and 1%, respectively (Sedgley and Lennan 2004).

It is feasible that if inoculation of enterococci occurs during initial root canal therapy or due to subsequent microleakage these microorganisms will persist in the root canal far after other bacteria have died, due to their fastidious nature(Figdor 2003). *E. faecalis* was shown to be capable of withstanding high pH changes as well as prolonged periods of little to no nutrients (Orstavik and Haapasalo 1990). Despite its adept ability to accommodate to the surrounding environment, *E. faecalis* does not seem to exhibit significant pathogenic qualities within the root canal system as a monoinfection.

In a monkey study in which enterococci were inoculated into monkey teeth only 2 of 9 cases developed radiographically-demonstrable periradicular lesions within six months (Fabricius et al. 1982). Closer examination of the possible mechanism of interaction between *E. faecalis* and the human immune cells revealed that inflammatory mediators responsible for osteolysis were actually decreased in the presence of these bacteria. Sonicated *E. faecalis* extracts were exposed to human T cells and the amount of interleukin-2 and Interleukin-4 production were measured (Ho-Hyun et al. 2004). Enterococcus decreased these immune mediators. According to this premise the presence of *E. faecalis* should decrease the incidence of periapical bone destruction. This mechanism is consistent with the findings of our study where *E. faecalis* was found to be associated with the lack of periapical breakdown.

E. faecalis alone does not appear to be a major player in the development of periapical disease but it may have an indirect role on other bacteria that may be detrimental to periapical health. Recently it was shown that *E. faecalis* may indirectly increase the survival and the pathogenicity of other bacteria in the root canal system (Moller and Fabricius 2004).

The unprecedented findings of this study belie the question: “Are *Enterococcus* spp. important in periradicular pathology or merely opportunistic bystanders?”. The results of this study may serve as a means of redirecting efforts away from just the eradication of enterococci and toward addressing the polymicrobial pathogens involved in periradicular pathosis.

Table 1. *Enterococcus faecalis* recovered at time of retreatment: culture studies.

Frerquency	Study
27%	Moller, Odontol Tidskr 1966
32%	Molander et al., IEJ 1998
38%	Sundqvist et al., OOO
56%	Peciuliene et al., JOE 2000
30%	Hancock et al., OOOOE
37%	Pinheiro et al., OMI 2003
0%	Cheung & Ho, IEJ 2001

Table 2. *E faecalis* recovered at time of retreatment: molecular studies

Frequency	Study
0%	Rolph et al., J Clin Micro 2001 (n=11)
77%	Siquera et al., OOOOE 2004 (n=22)
64%	Rocas et al., JOE 2004 (n=14)
23%	Fouad et al., OOOOE 2005 (n=37)

Table 3 Bacteria presence using a universal primer and lesion status

	Bacteria Present	Bacteria Absent	Total
Lesion Present	35	1	36
Lesion Absent	17	5	22
			58

Table 4: Bacterial presence using a universal primer in relation to lesion status and restoration quality.

		Bacteria Present	Bacteria absent	Totals
Lesion Present	Adequate Restoration	21	0	36
	Inadequate Restoration	14	1	
Lesion Absent	Adequate Restoration	9	3	22
	Inadequate Restoration	8	2	
				58

Table 5 Bacterial presence in relation to restoration quality and obturation quality.

		Lesion Present		Lesion Absent	
		Good Restoration	Poor Restoration	Good Restoration	Poor Restoration
Bacteria Present	Adequate Obturation	11	9	5	4
	Poor Obturation	10	4	5	4
Bacteria Absent	Adequate Obturation	0	1	0	2
	Poor Obturation	0	0	3	0

Table 6: Clinical parameters of cases in which *Enterococcus* spp. was identified:

Lesion Status	Quality of Restoration	Quality of Obturation	Peri-operative symptoms	Number of <i>Enterococcus</i> spp.-positive cases
Present	Adequate	Adequate	Absent	1
Present	Adequate	Inadequate	Present	1
Absent	Adequate	Adequate	Absent	1
Absent	Adequate	Inadequate	Absent	2
Absent	Inadequate	Adequate	Present	2

Method: Neighbor Joining; Best Tree; tie breaking = Systematic
 Distance: Kimura 2-parameter; Gamma correction = Off
 Transition:Transversion Ratio = Estimate (Av. = 0.96);
 Gaps distributed proportionally

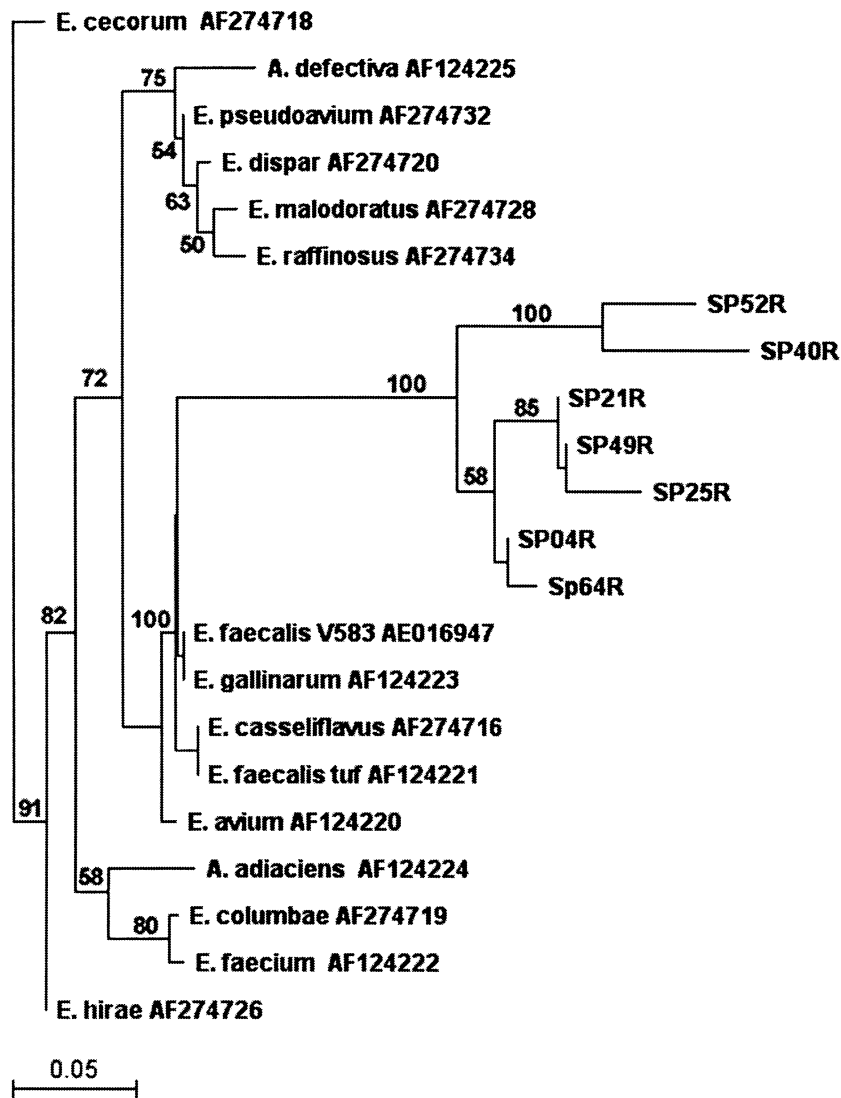


Figure Legend:

Figure 1: Dendrogram showing phylogenetic analysis of seven positive *Enterococcus* spp. specimens. This diagram represents the accepted method for describing the evolutionary relationship of organisms based on their DNA profiles. It is used to determine relatedness of organisms and thus aid in their identification. The numbers denote a boot strap analysis with 100 repetitions, which represent the chances that these phylogenetic relationships are absolute. The two genera represented are *Enterococcus* and *Abiotrophia*.

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